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(54) Title: METHODS FOR CHONDROCYTE GROWTH AND DIFFERENTIATION

(57) Abstract

The invention provides low density seeded chondrocytes (LDSC's) and methods for producing cultures of LDSC's. LDSC cultures are characterized by increased rates and levels of cellular proliferation and differentiation as compared to control cultures. The invention also provides methods of cartilage repair which utilize LDSC's. It also provides LDSC implantation methods, which methods involve implantation of cells into a cartilage defect *in vivo* at densities similar to those employed to establish LDSC cultures *in vitro*.

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METHODS FOR CHONDROCYTE GROWTH AND DIFFERENTIATION

Cross-Reference to Related Applications

This application is related to and claims the benefit of provisional application serial number 60/048,585 filed June 4, 1997.

Technical Field of the Invention

This invention relates to compositions and methods to enhance the growth and differentiation of chondrocytes. More specifically, this invention relates to the discovery of an inverse correlation between the initial cell density at which chondrocytes are seeded and their proliferative and differentiative capacity. Thus, the invention provides low density seeded chondrocytes characterized by increased rates and levels of cellular proliferation and differentiation. The invention also provides methods for generating low density seeded chondrocytes *in vitro* and *in vivo*. It also provides methods to enhance the growth and differentiation of chondrocytes implanted *in vivo* into sites of cartilage damage, thus accelerating tissue repair. This invention also provides methods of cartilage repair which utilize low density seeded chondrocytes according to this invention.

BACKGROUND OF THE INVENTION

Articular cartilage, found at the end of articulating bones, is a specialized tissue responsible for elasticity, resistance to compressive forces, and the smooth gliding that is characteristic of healthy joint function. Cartilage is composed of chondrocytes embedded in a hydrated extracellular matrix rich in collagens (predominantly type II) and

proteoglycans (predominantly aggrecan). The matrix macromolecules are synthesized by the chondrocytes.

In vitro systems for the study of normal chondrocyte proliferation and function have been hampered by the tendency of chondrocytes to "de-differentiate" during expansion in monolayer culture. This "de-differentiation" is evidenced by a flattened fibroblastic shape and an altered gene expression profile. Specifically, chondrocytes expanded in monolayer culture express elevated levels of type I collagen (a matrix molecule not normally produced by differentiated chondrocytes) and significantly reduced levels of type II collagen, aggrecan and proteoglycan (all markers for differentiated chondrocytes) as compared to the levels expressed during normal cartilage development.

One approach to maintain a differentiated chondrocyte phenotype involves growth in suspension cultures such as agarose gels and alginate beads. See, e.g., H. J. Hauselmann et al., "Synthesis and Turnover of Proteoglycans by Human and Bovine Adult Articular Chondrocytes Cultured in Alginate Beads", *Matrix*, 12:116-29 (1992). However, such suspension cultures are associated with significantly reduced levels of cellular proliferation and differentiation as compared to the chondrocyte cultures of this invention.

Improved methods for chondrocyte growth and differentiation are also desirable in the field of *in vivo* tissue repair. For example, one of the most promising procedures reported for treatment of cartilage defects involves autologous transplantation of chondrocytes. In this procedure, a biopsy of healthy cartilage is taken from the femoral condyle of a patient suffering from an injury-induced defect to the femoral condyle. Chondrocytes released from the biopsy are expanded in monolayer culture *in vitro,* during which time they "de-differentiate." They are then re-implanted into the cartilage defect of the donor.

The generation of what is apparently normal hyaline cartilage in a defect after autologous transplantation indicates that the chondrocytes which have de-differentiated during expansion *in vitro*, redifferentiate in the cartilage defect into which they are transplanted. Thus, enhancement of chondrocyte growth and differentiation is an important objective for improvement of cell-based tissue repair procedures.

SUMMARY OF THE INVENTION

This invention is based on the discovery that the seeding density of human articular chondrocytes has a profound effect on the degree of cell proliferation and differentiation. Specifically, previously reported methods for *in vitro* chondrocyte suspension culture and *in vivo* implantation of chondrocytes involved seeding the cells at densities similar to those observed in adult cartilage, i.e., at at least 1×10^6 cells/ml or greater. The inventors herein have discovered that an inverse correlation exists between cell seeding density and the levels of proliferation and differentiation of the cells. Thus, chondrocytes seeded at a density 100 times lower than the lowest densities previously used, i.e., at 1×10^4 cells/ml as compared to 1×10^6 cells/ml, had a much higher rate of proliferation. Unexpectedly, this high rate of proliferation was accompanied by an increase in differentiation, manifested by higher expression levels of type II collagen and proteoglycans, including aggrecan, in the expanded cultures.

This effect was particularly unexpected in view of observations made during the study of monolayer cultures. In monolayer cultures, type II collagen expression is rarely observed unless cells approach very high density conditions, i.e., confluence or post-confluence.

Thus, in one embodiment, the invention provides low density seeded chondrocytes (hereinafter "LDSC's") characterized by increased rates and levels of cellular proliferation and differentiation. In *in vitro* cultures, these increased rates and levels of cellular proliferation and differentiation are accompanied by the formation of colonies consisting of clonal expansions of single cells and deposition of extracellular matrix materials.

In additional embodiments, this invention provides methods for producing LDSC's *in vitro* and *in vivo*. The *in vitro* methods involve seeding chondrocytes into suspension culture at a density of less than 1×10^6 cells/ml or cells/cc^3 of suspension material (hereinafter referred to as "cells/ml"). In one aspect of this embodiment, the cells are seeded at a density of between 5×10^5 and 1×10^6 cells/. More preferably, the cells are seeded at a density of between 1×10^5 and 5×10^5 cells/. Even more preferably, the cells are seeded at density of between 5×10^4 and 1×10^5 cells/. Most preferred is to seed the cells at a density of between 1×10^4 and 5×10^4 cells/ml. The cells may also be seeded at a density of less than 1×10^4 cells/ml.

The *in vivo* methods of producing LDSC's according to this invention are also referred to as "LDSC implantation methods." These methods involve seeding or implantation of chondrocytes into cartilage defects *in vivo* at specific cell densities based on the surface area of the defect requiring repair. Specifically, these methods comprises the steps of: (a) determining the surface area of the cartilage defect, (b) determining the number of chondrocytes which will correspond to a desired density of cells per/cm^2 of the defect, and (c) implanting that number of chondrocytes into the cartilage defect. In one aspect of this embodiment, the cells are implanted into the defect site at a density between 5×10^5 and 1×10^6 cells/cm^2 of defect. In another aspect of this embodiment, the cells are

implanted at a density between 1 x 10^5 and 5 x 10^5 cells/cm^2 of defect. Preferably, the cells are implanted at a density between 1 x 10^5 and 3 x 10^5 cells/cm^2 of defect. In another aspect of this embodiment, the cells are implanted at a density between 1 x 10^4 and 1 x 10^5 cells/cm^2 of defect. Preferably, the cells are implanted at a density between 1 x 10^4 and 5 x 10^4 cells/cm^2 of defect. In yet another aspect, the cells are implanted at a density of less than 1 x 10^4 cells/cm^2 of defect.

The chondrocytes to be implanted may be freshly harvested from cartilage or may be derived from monolayer or suspension cultures. They may also be LDSC's produced *in vitro* according to this invention.

In additional embodiments, this invention provides methods to enhance the rate of cartilage repair which comprise the step of producing LDSC's in a bio-degradable matrix or matrix forming material and implanting the LDSC matrix culture into a cartilage defect, either without prior chondrocyte expansion within the matrix *in vitro* or after chondrocyte expansion within the matrix *in vitro*.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows levels of type I collagen, type II collagen and aggrecan RNA expression (normalized to GAPDH expression) and typeII/typeI expression in experiment CDA3 after 2 weeks of alginate culture seeded with 3 different densities of chondrocytes.

Figure 2 shows results of the same assays performed after 4 weeks of culture.

Figure 3 shows RNA expression levels in experiment CDA4 after 4 weeks of alginate culture seeded with three different densities of chondrocytes.

Figure 4 quantitates cell proliferation (based on DNA content) after four weeks of alginate culture for four different cell strains, each seeded at three different densities.

Figure 5 quantitates the GAG levels after four weeks of alginate culture for four different cell strains, each seeded at three different densities.

Figure 6 (A-C) contains photographs of proliferating HC24 chondrocytes seeded at three different densities after 4 weeks in alginate culture. Specifically, Figure 6A shows a culture seeded at $1x10^4$ cells/ml of alginate, Figure 6B shows a culture seeded at $1x10^5$ cells/ml of alginate and Figure 6C shows a culture seeded at $1x10^6$ cells/ml of alginate. (For the extent of proliferation observed in these cells, see the DNA contents set forth in Figure 4). Each of the photographs was taken at the same magnification.

DETAILED DESCRIPTION OF THE INVENTION

In order that the invention may be more fully understood, the following definitions are provided.

As used herein, "cartilage" refers to a connective tissue that contains chondrocytes embedded in an extracellular matrix. Cartilage includes articular and meniscal cartilage. Normal articular cartilage may also be referred to as "hyaline" cartilage. The gel-like matrix of cartilage is composed largely of collagen fibrils (predominantly type II), various proteoglycans (predominantly aggrecan) and water.

As used herein, a "cartilage defect" includes both full-thickness chondral defects that extend to the subchondral bone and partial thickness defects. Full thickness defects may arise from severe trauma or during the late stages of degenerative diseases such as osteoarthritis. Partial thickness defects are restricted to the cartilage tissue and may present in a variety of ways, including as fissures, divots or clefts in the surface of the

cartilage or as cartilage fibrillations. Such defects have a variety of causes including physical trauma to the joint, mechanical derangements or the early stages of degenerative diseases such as osteoarthritis.

As used herein, "chondrocytes" refer to cells which, under appropriate conditions, produce the components of normal cartilage tissue. Chondrocytes useful in the compositions and methods of this invention may be from any animal, including human. For repair of human articular cartilage defects, mammalian chondrocytes are preferred. Human chondrocytes are most preferred.

Chondrocytes may be "differentiated" or "de-differentiated." As used herein, "differentiated" chondrocytes are chondrocytes that express type II collagen and aggrecan. As used herein, "de-differentiated" chondrocytes have reduced levels or no type II collagen expression and increased levels of type I collagen expression as compared to chondrocytes present in normal adult cartilage.

As used herein, "LDSC's" are chondrocytes seeded at an initial density of less than 1×10^6 cells/ml or cells/cc^3 of suspension material *in vitro* or less than 1×10^6 cells/cm^2 of surface area of cartilage defect *in vivo*.

As used herein, a "suspension material" is any material into which chondrocytes may be seeded and maintained *in vivo* or *in vitro*. Suspension materials do not include fibrin or tissue culture plastic on which monolayer cultures are maintained *in vitro*.

As used herein, an "LDSC culture" is an *in vitro* culture of chondrocytes produced by seeding fresh or passaged chondrocytes in a suspension material at an initial density of less than 1×10^6 cells/ml or cells/cc^3 of suspension material.

As used herein, a "control culture" is an *in vitro* suspension culture of chondrocytes produced by seeding fresh or passaged chondrocytes at an initial density of greater than or

equal to 1 x 10^6 cells/ml or cells/cc^3 of suspension material. Any control culture must also have at least 10 fold more cells/ml or cells/cc^3 of suspension material than an LDSC culture to which it will be compared.

As used herein, a "biodegradable matrix" is any material other than fibrin that may be populated by chondrocytes and degraded or resorbed *in vivo*.

As used herein, a "growth supporting medium" is any medium in which chondrocytes are able to proliferate and differentiate. Proliferation may be determined by a variety of methods including assays for increased cell number or DNA content. Differentiation may be determined by expression of type II collagen and aggrecan.

In one embodiment of this invention, LDSC's are provided. LDSC's are characterized by increased rates and levels of cellular proliferation and differentiation. In *in vitro* cultures, these increased rates and levels of cellular proliferation and differentiation are manifested by the formation of numerous colonies consisting of clonal expansions of single cells and deposition of extracellular matrix materials. Specifically, the colonies produced in LDSC cultures occur more frequently and are larger than the rare colony observed in control cultures. The cells in LDSC cultures also produce markedly increased levels of type II collagen RNA, aggrecan RNA, type II collagen protein and chondroitin-6 sulfate-containing molecules (chondroitin-6-sulfate is a glycosaminoglycan (GAG) present in proteoglycans), indicating a molecular phenotype consistent with that found in normal adult articular cartilage. Significantly, histologic analysis of the colonies produced in LDSC cultures reveals the presence of a mostly acellular matrix material encased by a layer of proliferating chondrocytes. *In vivo*, these increased rates and levels of cellular proliferation and differentiation of LDSC's result in enhanced repair of cartilage defects.

Determination of the rates and levels of cellular proliferation and differentiation in the LDSC cultures of this invention may be accomplished, when desired, by any of a variety of means well known to those of skill in the art. For example, cell number may be determined by, *inter alia*, cell counting, uptake of labeled nucleotides, or determination of DNA content. The differentiation state of the chondrocytes may be determined by examining the levels of type II collagen, the ratio of collagen type II/collagen type I expression or by determining the level of aggrecan, GAG or chondroitin-6-sulfate expression. Expression levels may be assessed by a variety of techniques including those which detect RNA and those which detect protein. These include, for example, immunohistochemistry, RNase protection, Northern blotting, quantitative RT-PCR or combinations thereof. Techniques for performing such assays are well known to those of skill in the art and kits are commercially available. See, for example, RNA Isolation: Handbook from RNeasy Total RNA Kit (Quiagen, Cat # 74104); Instruction Manual from MAXIscript T7 In Vitro Transcription Kit (Ambion, Cat # 1314); and Instruction Manual from HybSpeed RPA Kit (Ambion, Cat # 1412).

The LDSC's of this invention and cultures containing them have a variety of uses. For example, they are useful to study the physical, biological and chemical processes involved in chondrocyte growth, development, differentiation and function. They are also useful systems to analyze the effects of various agents which may accelerate, antagonize, or otherwise affect those processes. As the chondrogenic phenotype is easily identifiable, they are also useful as controls for the differentiation state of a given set of cells. In addition, they provide a ready source of proliferating chondrocytes and colonies of chondrocytes for cell-based methods of tissue repair *in vivo*. Finally, because LDSC's are characterized by increased rates and levels of cellular growth and differentiation, they are

well suited for systems in which it is desirable to genetically modify chondrocytes by, e.g., introduction of heterologous nucleic acid molecules, either for the purposes of *in vitro* culture of for the purposes of implantation *in vivo*.

In another embodiment of this invention, methods of producing LDSC cultures *in vitro* are provided. These methods comprise the step of seeding chondrocytes in suspension culture *in vitro* at a density of less than 1×10^6 cells/ml and allowing the cells to proliferate in a growth supporting medium for at least 48 hours. Preferably, the cells are allowed to proliferate for a week or more. A preferred density for seeding is between 5×10^5 and 1×10^6 cells/ml. More preferably, the cells are seeded at a density between 1×10^5 and 5×10^5 cells/ml. Even more preferably, the cells are seeded at density between 5×10^4 and 1×10^5 cells/ml. Most preferred is to seed the cells at a density between 1×10^4 and 5×10^4 cells/ml. The cells may also be seeded at densities of less than 1×10^4. All of the above densities are believed to be significantly lower than those utilized in other reported methods of suspension chondrocyte culture, which methods have utilized densities which approximate the physiological density of chondrocytes in cartilage *in vivo*.

The LDSC's may be expanded *in vitro* until a desired cell number is reached. We have expanded cultures for over three months without observing any reduction in the levels of chondrocyte proliferation or differentiation. The continued deposition of extracellular matrix materials during long-term culture indicates that LDSC chondrocytes are able to participate in the tissue repair process for extended periods of time.

As is evidenced by the examples to follow, the rates and levels of LDSC proliferation and differentiation are inversely correlated to the initial seeding density. However, the skilled artisan will also understand that the lower the initial density of a

culture, the higher the number of cell divisions required to achieve a given cell number. Thus, given the teachings set forth herein, the skilled artisan, mindful of well known parameters such as senescence, nutrient requirements, etc., may select among various combinations of seeding densities and culture conditions in order to achieve rates and levels of growth and differentiation that are desired for a particular purpose.

LDSC's according to this invention may be produced using primary chondrocytes (i.e., those freshly harvested from cartilage) or using chondrocytes obtained from low passage number *in vitro* cultures. Primary chondrocytes may be obtained from articular cartilage of an animal by any of a variety of techniques known to those of ordinary skill in the art, e.g., by sequential enzyme digestion. Useful enzymes for this purpose include, but are not limited to collagenase followed by trypsin-collagenase; protease followed by collagenase and hyaluronidase followed by collagenase-trypsin.

Due to the practical considerations of quickly obtaining large numbers of cells with which to seed the LDSC cultures of this invention, we currently prefer to expand primary chondrocytes for a short period of time in monolayer culture prior to initiating an LDSC culture. Specifically, we seed primary chondrocytes at approximately 2000 cells/cm^2 of tissue culture plastic and passage (1:20) at 7 day intervals until the desired cell number is reached. We typically use 3rd passage chondrocytes to initiate the LDSC cultures.

LDSC's may be produced by seeding chondrocytes in any of a variety of mediums useful to support chondrocyte growth and differentiation. Such growth supporting mediums typically comprise basal medium supplemented with vitamins, inorganic salts, hormones and growth factors necessary for appropriate cell proliferation and function. Examples of useful mediums include, without limitation, DME with 10% fetal bovine serum (FBS) + ascorbate +pyruvate or DME/Ham's F12 with 10% FBS + ascorbate. Also

known to the ordinarily skilled artisan in this field are defined mediums useful to support chondrocyte growth and differentiation. See, e.g., PCT application WO 98/04681, incorporated herein by reference.

The LDSC's of this invention may be produced utilizing any suspension culture other than fibrin. A variety of materials with which to prepare suspension cultures are available to those of skill in the art including, without limitation, alginate, agarose, various collagen sponges of differing porosities, other polymerized materials such as PLA and PGA mesh, and HA. Most preferred are alginate cultures, which may be prepared according to methods well known in the art. See, e.g., Guo et al., *Conn. Tiss. Res.*, 19:277-297 (1989), incorporated herein by reference. The use of fibrin cultures is not recommended, as it does not appear to support efficient differentiation of chondrocytes seeded at low densities.

In another embodiment of this invention, LDSC's produced *in vitro* are used to enhance the rate of tissue repair in a cartilage defect *in vivo*. Specifically, in cell based methods of tissue repair, new cartilage tissue is generated as a result of implanting chondrocytes into a selected defect site *in vivo*. LDSC cultures prepared according to this invention provide a ready source of chondrocytes for such procedures. For this use, LDSC's may be implanted as isolates of cells separated from the suspension culture or may be implanted without prior separation. For example, when alginate is used as the suspension culture, the cells may be released from the alginate by addition of citrate or the cells may be implanted while still suspended in the alginate beads. In addition, for the purposes *of in vivo* implantation, the LDSC's may be separated from the matrix materials deposited in the colonies, e.g., by enzymatic digestion, or entire colonies may be implanted, containing cells and an inner core of extracellular matrix. In any case, the use

of LDSC's accelerates the tissue repair process *in vivo* due to their increased capacity for growth and differentiation.

A variety of cell-based methods of cartilage repair are known to those of skill in the art. (See, e.g., T. Minas and S. Nehrer, "Current Concepts in the Treatment of Articular Cartilage Defects", Orthopedics, 20:525-538 (1997) and H. Breinan et al., "Histological evaluation of the course of healing of canine articular cartilage defects treated with cultures of autologous chondrocytes" *Tissue Engineering*, 4:101-114 (1998). For example, one such method in widespread use in the United States is autologous chondrocyte implantation (ACI). ACI involves arthroscopic removal from the patient of a small sample of healthy cartilage, expansion of the chondrocytes in monolayer cultures *in vitro*, and re-implantation of the expanded autologous cells. See, M. Brittberg et al., "Treatment Of Deep Cartilage Defects In The Knee With Autologous Chondrocyte Implantation", *N. Eng. J. Med.*, 331:889-95 (1994), incorporated herein by reference. In the method currently in use, a fixed number of cells (approximately 12 x 10^6) are implanted into defects having surface areas which average 4.5 cm^2. The substitution of LDSC's produced *in vitro* according to the methods of this invention for the de-differentiated cells currently used in this procedure could be readily accomplished by the skilled artisan.

Other cell based methods of tissue repair in which LDSC's may be useful include those which utilize a biodegradable matrix to deliver or maintain the chondrocytes *in vivo*. Such matrices may be formed from a variety of materials and by numerous methods known to those of skill in the art. See, e.g., U.S. patents 5,270,300 and 5,368,858. See also M. Zimber et al., *Tissue Engineering*, 1:289-300 (1995); D. Robinson et al., *Calcif. Tissue Int.*, 46:246-53 (1990); S. Nehrer et al., *Biomaterials*, 18:769-76 (1997); A. Sams et al., *Osteoarthritis and Cartilage*, 3:61-70 (1995); and A. Sams and A. Nixon, *Osteoarthritis*

and Cartilage, 3:47-59 (1995). Materials which may be useful include, without limitation, collagen (e.g., collagen sponges), agarose, gelatin beads or sponges, HA, PGA and other materials within which chondrocytes may be seeded and proliferate.

In another embodiment of this invention, methods of producing LDSC's *in vivo* are provided. These methods, also referred to as "LDSC implantation methods," involve implantation of chondrocytes into a site of cartilage defect, either directly or in the form of a matrix, at specific cell densities based on the surface area of the defect requiring repair. Specifically, the method comprises the steps of: (a) determining the surface area of the cartilage defect, (b) determining the number of chondrocytes which will correspond to a desired density of cells per cm^2 of the defect, and (c) implanting that number of chondrocytes into the cartilage defect. In one aspect of this embodiment, the cells are implanted into the defect site at a density between 5×10^5 and 1×10^6 cells/cm^2 of defect. In another aspect of this embodiment, the cells are implanted at a density between 1×10^5 and 5×10^5 cells/cm^2 of defect. Preferably, the cells are implanted at a density between 1×10^5 and 3×10^5 cells/cm^2 of defect. In another aspect of this embodiment, the cells are implanted at a density between 1×10^4 and 1×10^5 cells/cm^2 of defect. Preferably, the cells are implanted at a density between 5×10^4 and 1×10^5 cells/cm^2 of defect. More preferably, the cells are implanted at a density between 1×10^4 and 5×10^4 cells/cm^2 of defect. In yet another aspect, the cells are implanted at a density of less than 1×10^4 cells/cm^2 of defect.

The chondrocytes to be implanted may be freshly harvested from cartilage or may be derived from monolayer or suspension cultures. LDSC implantation methods may also utilize LDSC's produced *in vitro* according to the methods of this invention.

Cartilage defects may be detected and measured by visual examination during arthroscopic or open surgery or by CAT scan, x-ray, MRI or any other of a number of procedures known to those of skill in the art. Diagnostic markers for cartilage pathology or loss of cartilage tissue are also available.

The LDSC implantation methods of this invention may be utilized as a part of any cell-based method of cartilage repair that the skilled artisan wishes to practice. Thus, currently approved procedures such as ACI as well as experimental procedures such as those designed to enhance delivery, maintenance or development of the implanted cells (e.g., procedures which utilize various delivery vehicles, matrices, growth or differentiation factors, biological glues, and other features designed to accelerate the repair process) are all readily amenable to adaptation so as to employ the teachings of this invention. Of course, the skilled artisan will understand that the optimal density at which to implant the cells may be affected by the particular steps of the repair method being utilized. Therefore, once within the ranges provided herein, the particular density to incorporate into any given method of tissue repair should be selected in conjunction with whatever model system(s) are employed to study the efficacy of that method.

One of skill in the art will also understand that any matrix or medium used to support the chondrocytes, either during LDSC culture *in vitro* or for the purposes of *in vivo* implantation, may optionally contain various growth factors, differentiation factors, or chemotactic agents which aid in chondrocyte growth or differentiation or accelerate or modulate tissue repair. Such factors include members of the transforming growth factor β (TGFβ) family, members of the fibroblast growth factor (FGF) family, members of the insulin-like growth factor (IGF) family and bone morphogenic proteins (BMP's).

One of skill in the art will also understand that, if LDSC's are used in a method of *in vivo* tissue repair which employs implantation of heterologous cells (i.e., cells derived from an animal, including human, other than the recipient), it may be desirable to treat the cells prior to implantation in order to decrease their antigenicity. Alternatively, a recipient of a heterologous implant may be treated prior to and/or subsequent to implantation in order to dampen the immune response against the implanted cells. Those of skill in the art of heterologous transplants are well versed in techniques useful to accomplish these ends.

The following examples are set forth in order that the invention described herein may be more fully understood. These examples are for illustrative purposes and are not to be construed as limiting this invention.

Example I: Production of LDSC's in Agarose

We isolated primary chondrocytes from goat articular cartilage by overnight digestion with 0.1% collagenase. The chondrocytes were harvested by centrifugation, seeded into monolayer culture at 2000 cells/cm^2 tissue culture plastic in DME (Gibco BRL, Gaithersburg, MD) + 10% FBS (Hyclone, Logan, Utah) and allowed to grow until approximately 80% confluent, at which time frozen stocks were prepared. Prior to seeding of LDSC cultures, aliquots of frozen stocks were thawed and expanded to 3rd passage. We then harvested the third passage, de-differentiated goat articular chondrocytes from monolayer culture and seeded them into 2% low melt agarose (FMC, Rockland, ME) at 45° C at densities of $1x10^4$, $5x10^4$, $1x10^5$, and $5x10^5$ cells/ml agarose. The agarose gels / cells were allowed to gel at room temperature for 1 hour, and then cultured for four weeks in 1:1 Dulbecco's Modified Eagle / Ham's F12 media (DME/F12) (Gibco BRL, Gaithersburg, MD) + 10% FBS at 37° C and 8% CO_2 with media changes every other day. After four

weeks, the agarose cultures were fixed with 10% formalin and stained with Safranin O (Sigma, St. Louis, MO), which stains for sulfated proteoglycan (orange). We documented colony size and morphology by photography.

Colony morphology and Safranin O stain showed an inverse correlation with seeding density. Specifically, the lower the seeding density, the larger the colonies observed (indicating a higher level of proliferation) and the greater the Safranin O staining (indicating a higher level of differentiation).

Example II: **Production of LDSC's in Alginate**

We obtained human articular chondrocytes from biopsies provided by National Disease Research Interchange (NDRI, Philadelphia, PA) and harvested the cells as in Example I except that a second digestion with collagenase-trypsin was performed for 4-6 hours. We expanded the cells in monolayer culture in DME + 10% FBS. We then harvested third passage, de-differentiated human articular chondrocytes from the monolayer culture and seeded them in 1.2% alginate beads (Kelco, Chicago, IL) according to a modification of the method of Guo et al. *Conn. Tiss. Res.*, 19:277-297 (1989). The cells were seeded at densities of $1x10^4$, $1x10^5$, and $1x10^6$ cells/ml alginate, and cultured in suspension for two and / or four weeks in DME/F12 + 10% FBS + 25 μg/ml ascorbate (Sigma) at 37° C and 8% CO_2, with media changes every other day. At the time of harvest (i.e. either at two or four weeks), the cells were released from the alginate beads by the addition of 55 mM sodium citrate (Sigma) and harvested by centrifugation.

We then assayed the cells for DNA content using Hoechst dye H33258 (Molecular Probes, Eugene, OR) and determined glycosaminoglycan (GAG) levels using a DMMB (1, 9-dimethly-methlylene blue) assay (Aldrich, Milwaukee, WI). In addition, we determined

the levels of type I collagen, type II collagen and aggrecan RNA using an RNase protection assay obtained from Ambion (Austin, TX) and performed according to manufacturer's instructions. We detected type II collagen protein expression and GAG levels by immunohistochemistry using antibodies directed against type II collagen or chondroitin-6-sulfate (Chemicon, Temecula, CA) and an immunoperoxidase based detection method from Vector Labs (Burlingame, CA).

We conducted three separate experiments, CDA-3, CDA-4, and CDA-5. In each experiment, cells were seeded at densities of $1x10^4$, $1x10^5$ and $1x10^6$ cells/ml alginate. In experiment CDA-3, cultures of chondrocyte strain HC36 were harvested after two and/or four weeks and expression of type I collagen, type II collagen and aggrecan RNA at each time point was determined by RNase protection. (Strain HC36 was derived from a 36 year old patient). The expression levels of type II collagen and aggrecan RNA were normalized by comparing to GAPDH RNA.

Experiment CDA-4 measured expression of type I collagen, type II collagen and aggrecan RNA in 4 week cultures of chondrocyte strain HC36. In this experiment, the proliferation rate of the cells was also determined using a Heochst assay for DNA content. The cultures were also assayed for differentiation using DMMB to detect GAG.

Experiment CDA-5 assayed cultures derived from donors of three different ages for chondrocyte proliferation rates (Heochst assay) and differentiation levels (DMMB). Specifically, HC24, HC31, and HC52, correspond to patients of ages 24, 31 and 52, respectively.

Figure 1 shows the results of experiment CDA-3 after 2 weeks in culture. As may be seen, the LDSC's in cultures seeded at 1 x 10^4 cells/ml (solid bars) expressed 24.2 times more type II collagen RNA, 2.3 times more aggrecan RNA, and a 19 fold higher ratio of

type II to type I collagen RNA than cells in control cultures seeded at 1 x 10^6 cells/ml. Similar results are obtained after four weeks in culture (Figure 2). The inverse correlation between initial seeding density and differentiation levels was reproduced in independent experiments measuring the same parameters. See, e.g., the results of CDA-4, set forth in Figure 3.

The LDSC's also had increased proliferation rates compared to control cultures. Specifically, Figure 4 shows the results obtained when chondrocytes from four different patients were seeded at densities of $1x10^4$, $1x10^5$ and $1x10^6$ cells/ml alginate and the extent of proliferation was determined by quantitating the total levels of DNA using the Hoechst assay. Over the four weeks of culture, HC24 cells seeded at 1 x 10^4 cells/ml increased their DNA content by 4.3 fold. In contrast, the control culture of HC24 cells seeded at 1 x 10^6 cells/ml had only a 1.4 fold increase in DNA content over the same time period. Thus, the proliferation rate in an LDSC culture derived from HC24 cells was 3 times higher than in the control culture of the same cells. Figure 4 also demonstrates that the increased proliferation rate of LDSC's is not dependent on the age of the donor. Thus, HC31 cells seeded at $1x10^4$ cells/ml had a proliferation rate more than 2.6 fold higher than control cells derived from the same donor; the proliferation rate of HC36 cells seeded at $1x10^4$ cells/ml was almost 6 fold higher than a control culture, and the proliferation rate of HC52 cells seeded at $1x10^4$ cells/ml was 2.7 fold higher than the control culture.

Figure 5 demonstrates the increased levels of proteoglycan expression consistent with increased differentiation in LDSC cultures. In order to normalize for differences in cell number, the GAG levels are set forth as ratios which take into account the DNA content in each sample. As seen in Figure 5, the cultures seeded at $1x10^4$ cells/ml had GAG levels 3.6 to 11 times higher than did control cultures prepared from the

corresponding donor. Again, the increased differentiation observed in LDSC cultures was not dependent on age and was inversely correlated to the initial seeding density. Specifically, proteoglycan expression in an LDSC culture derived from HC24 cells was 5.8 fold higher than in the control culture derived from the same donor; proteoglycan expression in an LDSC culture derived from HC31 cells was 4.8 fold higher than in the control culture derived from the same donor; proteoglycan expression in an LDSC culture derived from HC36 cells was 11 fold higher than in the control culture derived from the same donor; and proteoglycan expression in an LDSC culture derived from HC52 cells was 3.6 fold higher than in the control culture derived from the same donor.

Morphological analysis (by light microscopy) of colonies obtained after 4 weeks in alginate culture clearly demonstrates the inverse correlation between seeding density and the proliferation levels of the cells. Specifically, cells seeded at $1x10^4$ cells/ml clonally expand to form large, multicellular colonies. In contrast, cells seeded at $1x10^6$ cells/ml proliferated to a much lesser extent and the rare colony that occurred was much smaller.

An example of these results is set forth in Figure 6. Figure 6A is a photograph of a single colony obtained from a 4 week alginate culture of chondrocytes seeded at $1x10^4$ cells/ml. Figure 6B is the same magnification when the cells are seeded at $1x10^5$ cells/ml. Figure 6C shows a control culture seeded at $1x10^6$ cells/ml.

Histological analysis of LDSC colonies performed by sectioning through a parafin embedded alginate bead and H and E staining revealed the presence of a mostly acellular matrix material encased by a layer of proliferating chondrocytes. Safranin-O staining for GAGs also reveals the presence of matrix material surrounding the colony. In long term LDSC cultures (8 weeks or more), adjacent colonies may also begin to integrate with each other forming larger aggregates.

Example III: Use of LDSC's Produced *In Vitro* In Cell Based Methods of Tissue Repair

We first obtain a sample of healthy cartilage, isolate the chondrocytes and expand to 3rd passage in monolayer culture. We then prepare LDSC's in alginate culture as described in Example II by seeding third passage chondrocytes at 1 x 10^4 cells/ml in alginate and culturing until a desired cell number is reached. We then harvest the cells for implantation into the cartilage defect of the patient. The cells may be implanted in a variety of forms. In the first implantation method, the cells/colonies are released from the alginate beads by addition of three volumes of 55 mM sodium citrate. After centrifugation, the cells/colonies are washed twice with PBS and resuspended in DME for implantation. In the second implantation method, the alginate beads are implanted directly, without releasing or disrupting the cells/colonies. In the third implantation method, the cells are released from the alginate and from the extracellular matrix materials deposited during culture by treatment with sodium citrate and collagenase and /or trypsin as in Examples I and II.

We implant the LDSC's in any of the forms described above using the ACI procedure set forth in Brittberg et al. Briefly, the chondral lesion is excised as far as the normal surrounding cartilage but not into the subchondral bone plate. We then inject 2.5 million to 5 million LDSC's into the area of the defect beneath a periosteal flap taken from the proximal medial tibia and sutured to the surrounding rim of the normal cartilage. Rehabilitation after surgery is initiated by active movement without weight bearing. Weight bearing is gradually introduced over the next eight weeks. Patients are evaluated every 8 to 12 weeks after implantation.

Example IV: **LDSC Implantation Methods**

We first perform diagnostic assays to determine the surface area of the cartilage defect requiring repair. We then implant freshly harvested chondrocytes or dedifferentiated chondrocytes obtained from monolayer culture into the cartilage defect at various densities based on the surface area of the defect.

Specifically, we implant cells at densities between 5×10^5 and 1×10^6 cells/cm^2 of defect, between 1×10^5 and 5×10^5 cells/cm^2 of defect, between 1×10^5 and 3×10^5 cells/cm^2 of defect, between 5×10^4 and 1×10^5 cells/cm^2 of defect, between 1×10^4 and 5×10^4 cells/cm^2 of defect and at densities of less than 1×10^4 cells/cm^2 of defect. The implantation procedure is as set forth in Example III.

We then evaluate the rate of cartilage repair resulting from each of the implantation densities based on factors such as pain, swelling, locking and arthroscopic evaluation. We then select the implantation density which provides the most desired rate of cartilage repair.

Example V: **LDSC Implantation Methods Using LDSC's Produced *In Vitro***

We first perform diagnostic assays to determine the surface area of the cartilage defect requiring repair. We then remove a sample of healthy cartilage from a donor and prepare LDSC's in alginate culture as described in Example II by seeding at 1×10^4 cells/ml and culturing until a desired cell number is reached. We then implant the cells into the cartilage defect at various densities based on the surface area of the defect. The cells are implanted in any of the forms described in Example III.

The implantation procedure is as set forth in Example III, except that we implant cells at densities between 5 x 10^5 and 1 x 10^6 cells/cm^2 of defect, between 1 x 10^5 and 5 x 10^5 cells/cm^2 of defect, between 1 x 10^5 and 3 x 10^5 cells/cm^2 of defect, between 5 x 10^4 and 1 x 10^5 cells/cm^2 of defect, between 1 x 10^4 and 5 x 10^4 cells/cm^2 of defect and at densities of less than 1 x 10^4 cells/cm^2 of defect. After allowing the recipients to rehabilitate, we evaluate the rate of cartilage repair resulting from each of the implantation densities based on factors such as swelling, locking and arthroscopic evaluation. We then select the implantation density which provides the most desired results.

Example VI: **Use of a Biodegradable Matrix to Implant LDSC's Produced in Suspension Culture *In Vitro***

We prepare LDSC's by seeding fresh or third passage chondrocytes in alginate at 1 x 10^4 cells/ml as in Example II. After culturing for various time periods between 2 days and 4 weeks, the cells are released from alginate by addition of sodium citrate as in Example III and seeded into a commercially available collagen sponge. We then implant and secure the sponge in a cartilage defect of an animal *in vivo* and allow the recipient to rehabilitate as in Example III. We then evaluate the recipients as in previous examples.

Example VI: **Use of a Biodegradable Matrix to Produce and Implant LDSC's**

We prepare LDSC's by seeding fresh or third passage chondrocytes into a collagen sponge at 1 x 10^4 cells/ml. After culturing for various time periods between 2 days and 4 weeks, or, without such culture, the sponge is implanted into a cartilage defect *in vivo* of an anial in vivo and secured. The recipient is allowed to rehabilitate and evaluated as in previous examples.

We claim:

1. A method of producing a low density seeded chondrocyte culture (LDSC culture) *in vitro*, comprising the steps of:

(a) seeding chondrocytes in a suspension material at a density of less than 1×10^6 cells/ml; and

(b) allowing the cells to proliferate and differentiate for at least 48 hours.

2. The method according to claim 1, wherein the cells are seeded at a density between 1×10^5 to 5×10^5 cells/ml.

3. The method according to claim 1, wherein the cells are seeded at a density between 1×10^4 to 1×10^5 cells/ml.

4. The method according to claim 1, wherein the cells are seeded at a density of less than 1×10^4 cells/ml.

5. The method according to any one of claims 1-4, wherein the cells are seeded into a biodegradable matrix.

6. The method according to any one of claims 1-4, wherein the cells are seeded into alginate.

7. An LDSC culture produced by a method according to any one of claims 1-6.

8. A method of cartilage repair comprising the steps of:

(a) removing a sample of healthy cartilage from a donor animal;

(b) isolating chondrocytes from said cartilage;

(c) seeding said chondrocytes in a suspension material at a density of less than 1×10^6 cells/ml; and

(d) implanting said chondrocytes into a cartilage defect in a recipient animal *in vivo*.

9. The method according to claim 8, wherein said chondrocytes are cultured in said suspension material *in vitro* prior to implanting *in vivo*.

10. The method according to claim 9, wherein said suspension material is alginate.

11. The method according to claim 8 or 9, wherein said suspension material is a biodegradable matrix.

12. The method according to any of claims 8-11, wherein said donor animal is different from said recipient animal.

13. The method according to any of claims 8-11, wherein said recipient animal is human.

14. An LDSC implantation method comprising the steps of:

(a) determining the surface area of a cartilage defect in a recipient animal;

(b) determining the number of chondrocytes which will correspond to a desired density of cells/cm^2 of defect, which density is less than 1 x 10^6/cm^2 of said defect; and

(c) implanting that number of chondrocytes into said cartilage defect.

15. The method according to claim 14, wherein said chondrocytes are implanted at a density between 1 x 10^5 to 5 x 10^5 cells/ cm^2 of the defect.

16. The method according to claim 14, wherein said chondrocytes are implanted at a density between 1 x 10^4 to 1 x 10^5 cells/ cm^2 of the defect.

17. The method according to claim 14, wherein said chondrocytes are implanted at a density of less than 1 x 10^4 cells/cm^2 of the defect.

18. The method according to any one of claims 14-17, wherein said chondrocytes are seeded into a biodegradable matrix prior to implantation.

19. The method according to any one of claims14-17, wherein said chondrocytes are seeded into alginate prior to implantation.

20. The method according to any one of claims 14-17, wherein said chondrocytes are passaged chondrocytes derived from monolayer culture.

21. The method according to any one of claims 14-17, wherein said chondrocytes are LDSC chondrocytes derived from a suspension culture.

22. The method according to any one of claims 14-17, wherein said chondrocytes are derived from an animal other than said recipient animal.

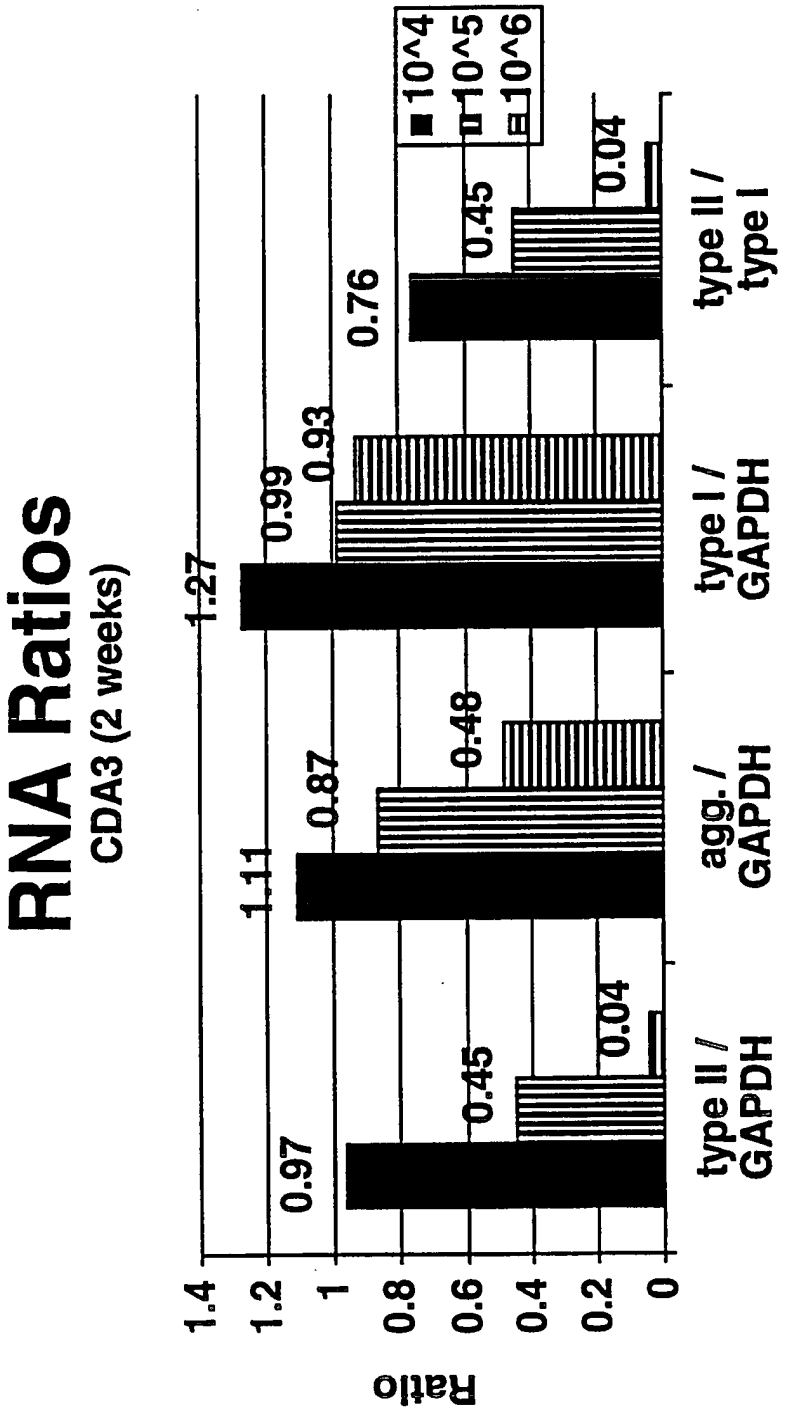
RNA Ratios
CDA3 (2 weeks)
Ratio
1.4
1.2
1
0.8
0.6
0.4
0.2
0
10^4
10^5
10^6
1.27
0.99
0.93
0.76
0.45
0.04
1.11
0.87
0.48
0.97
0.45
0.04
type II / type I
type I / GAPDH
agg. / GAPDH
type II / GAPDH
RNA Marker

Figure 1

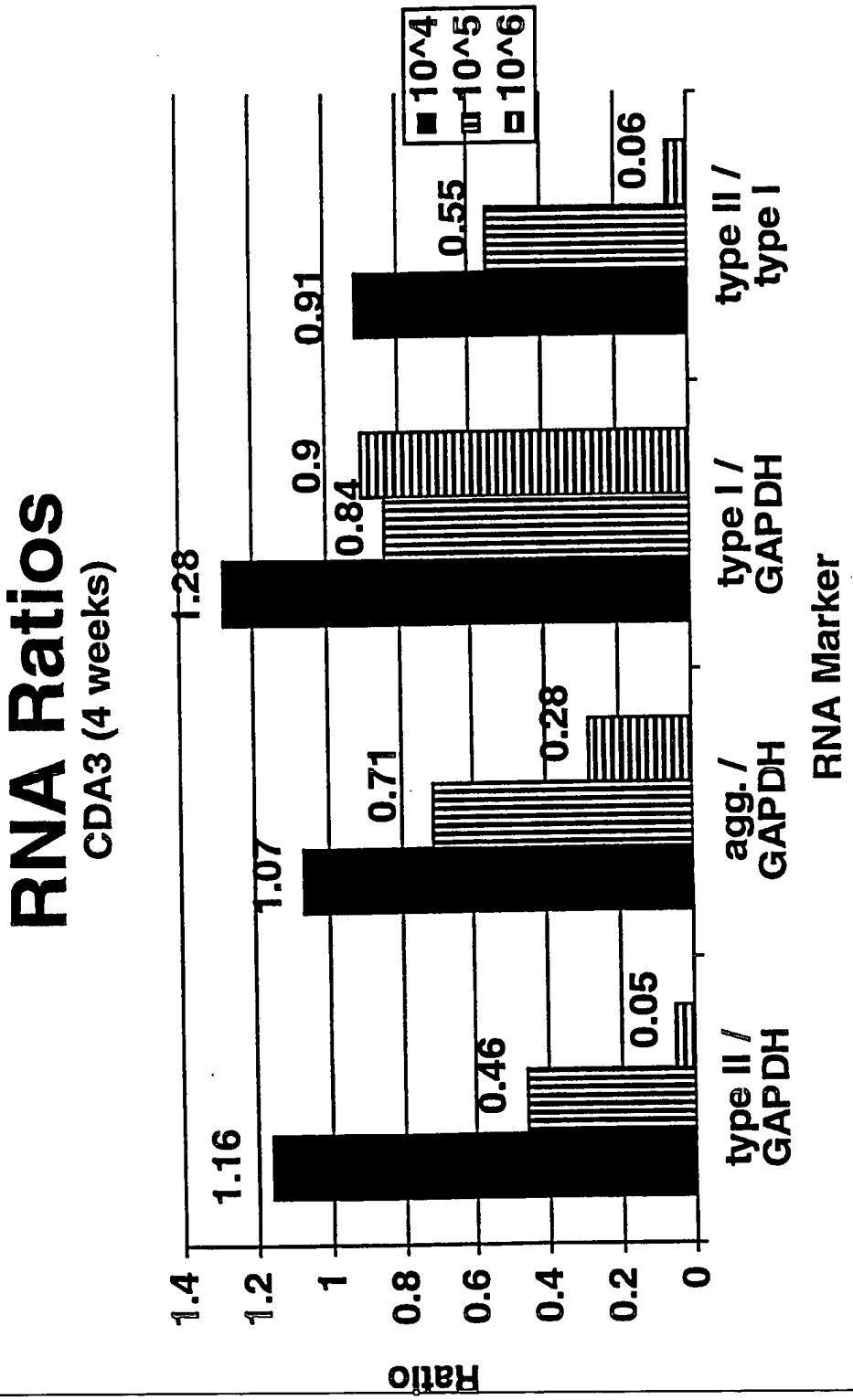
RNA Ratios
CDA3 (4 weeks)
10^4
10^5
10^6
Ratio
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1.16
0.46
0.05
1.07
0.71
0.28
1.28
0.84
0.9
0.91
0.55
0.06
type II / GAPDH
agg. / GAPDH
type I / GAPDH
type II / type I
RNA Marker

Figure 2

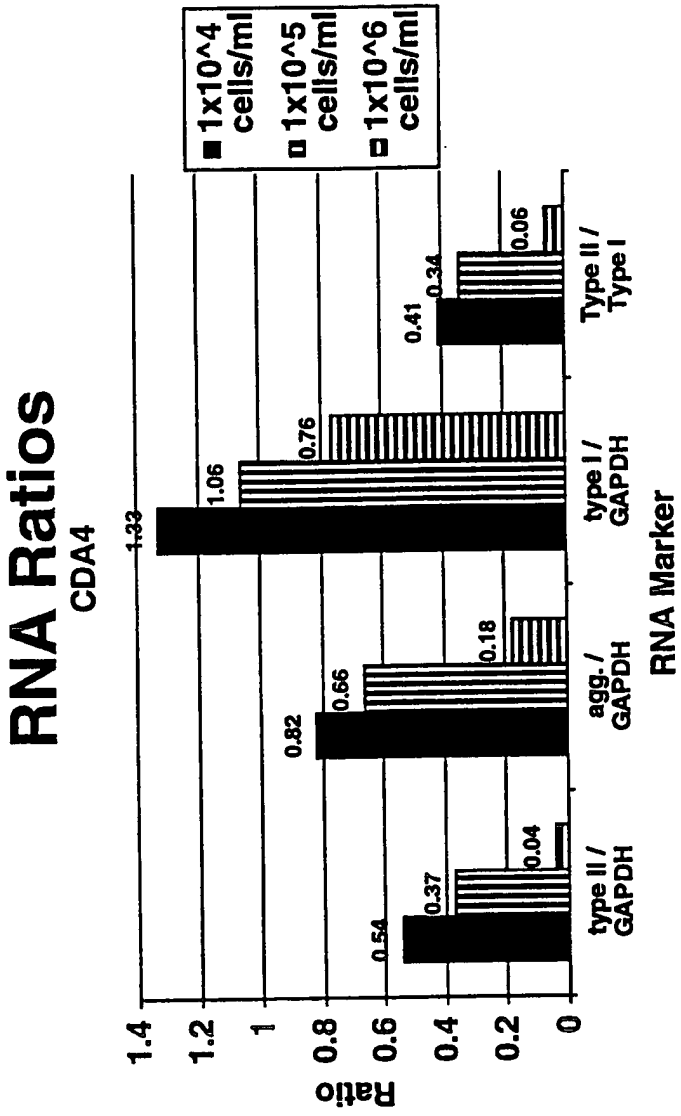
RNA Ratios
CDA4
1x10^4 cells/ml
1x10^5 cells/ml
1x10^6 cells/ml
Ratio
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0.54
0.37
0.04
0.82
0.66
0.18
1.33
1.06
0.76
0.41
0.34
0.06
type II / GAPDH
agg. / GAPDH
type I / GAPDH
Type II / Type I
RNA Marker

Figure 3

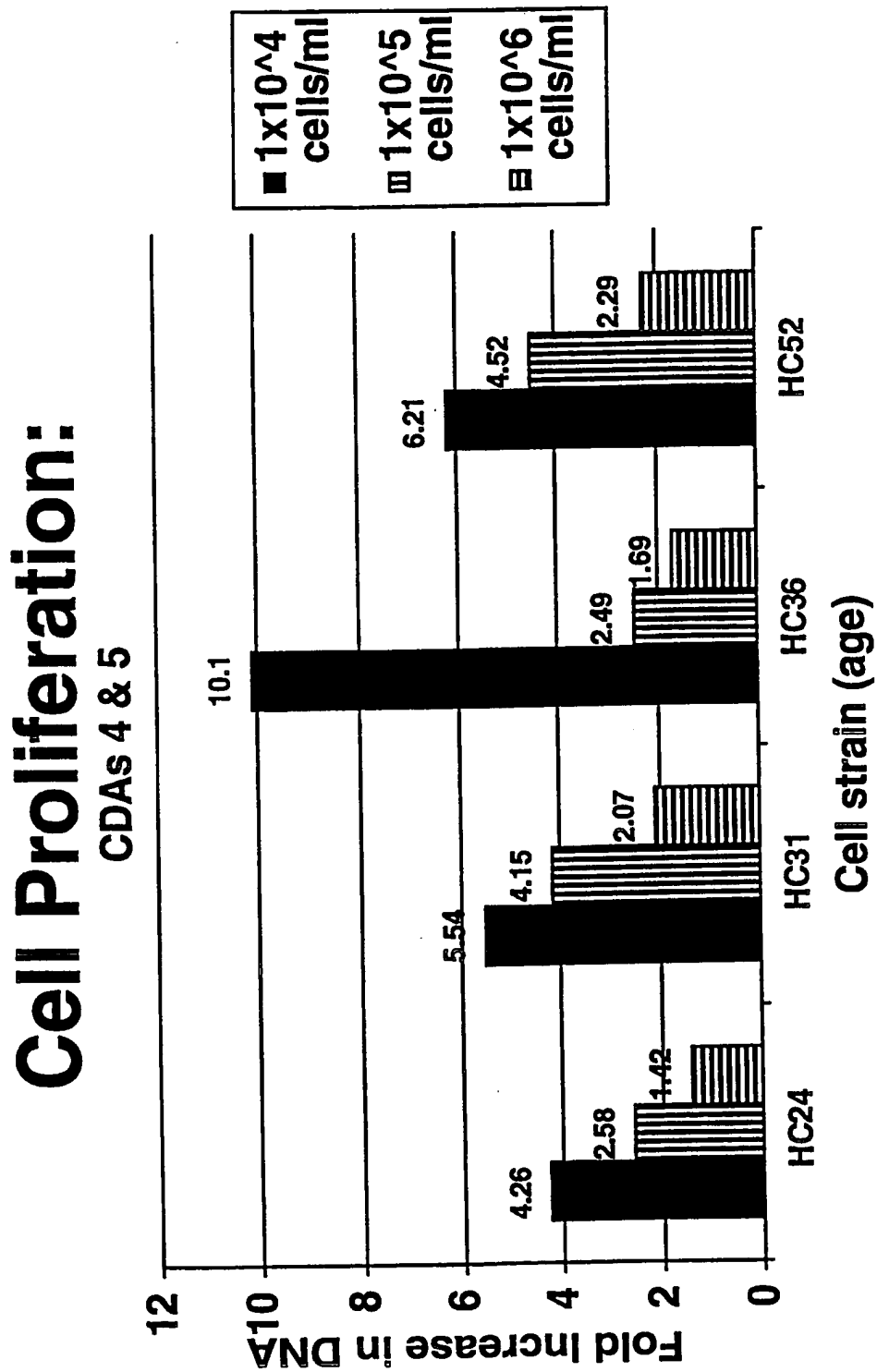
Cell Proliferation:
CDAs 4 & 5
1x10^4 cells/ml
1x10^5 cells/ml
1x10^6 cells/ml
Fold Increase in DNA
0
2
4
6
8
10
12
4.26
2.58
5.54
4.15
10.1
2.49
6.21
4.52
HC24
HC31
HC36
HC52
Cell strain (age)

Figure 4

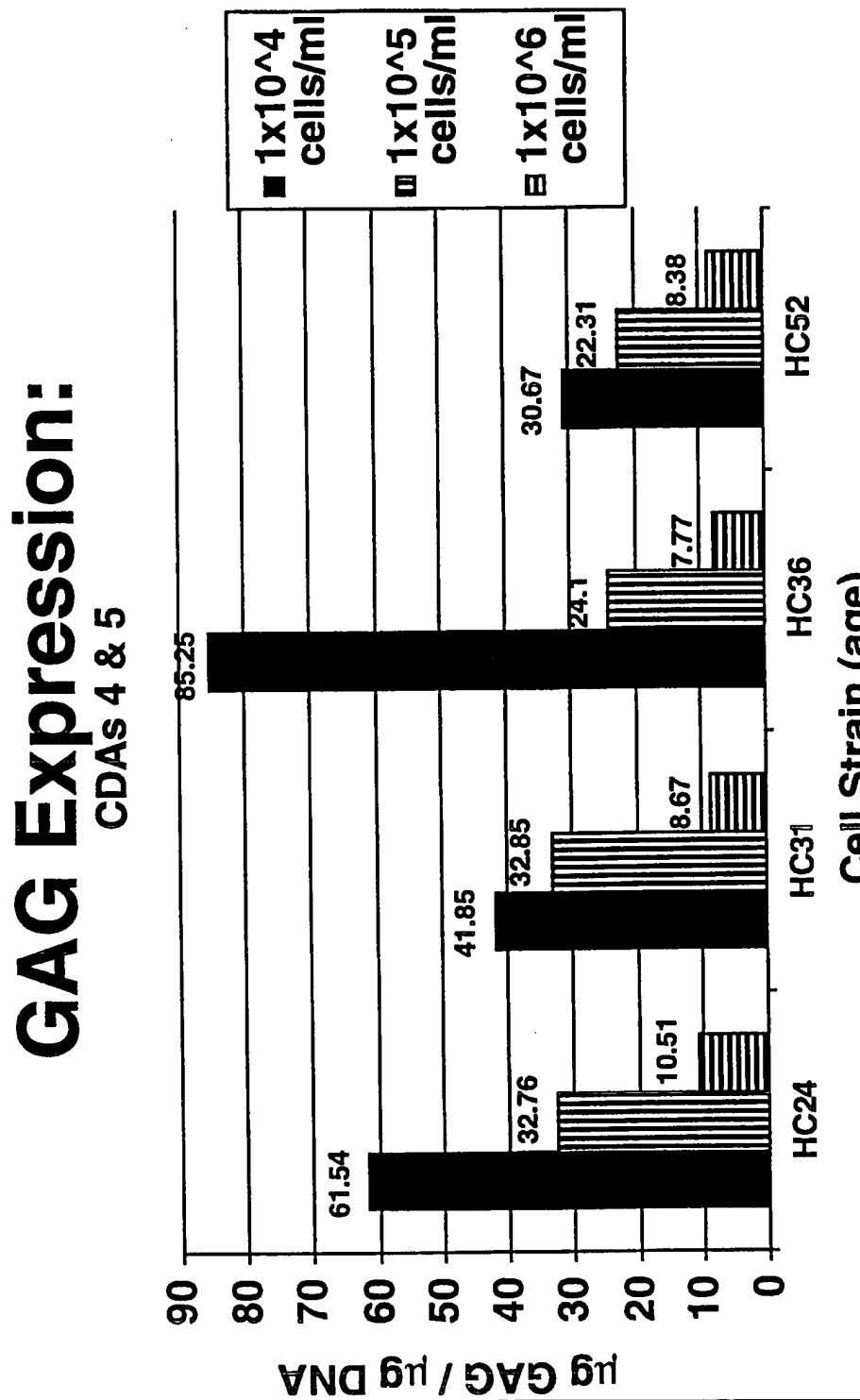
GAG Expression:
CDAs 4 & 5
1x10^4 cells/ml
1x10^5 cells/ml
1x10^6 cells/ml
µg GAG / µg DNA
0
10
20
30
40
50
60
70
80
90
61.54
32.76
10.51
41.85
32.85
8.67
85.25
24.1
7.77
30.67
22.31
8.38
HC24
HC31
HC36
HC52
Cell Strain (age)

Figure 5

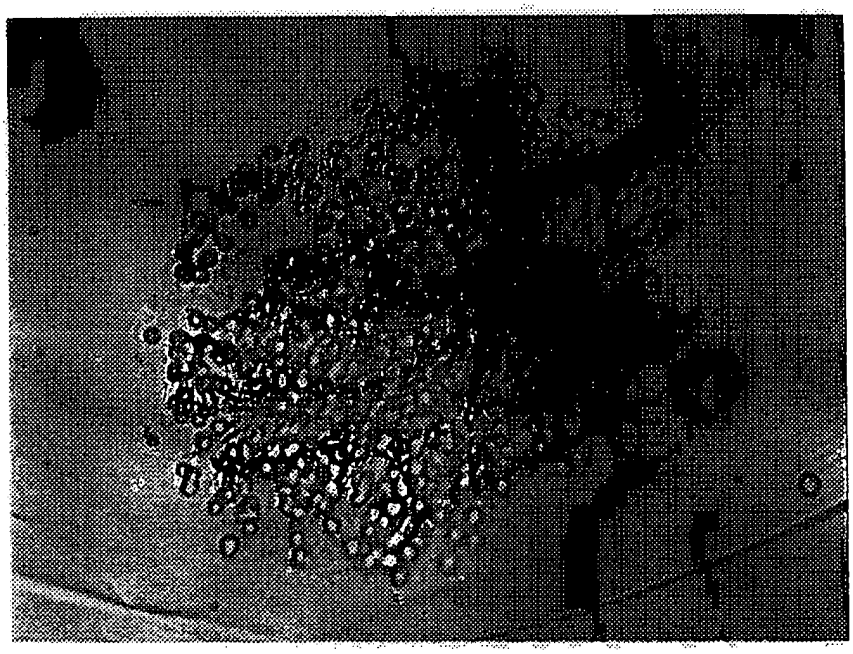

FIG. 6a

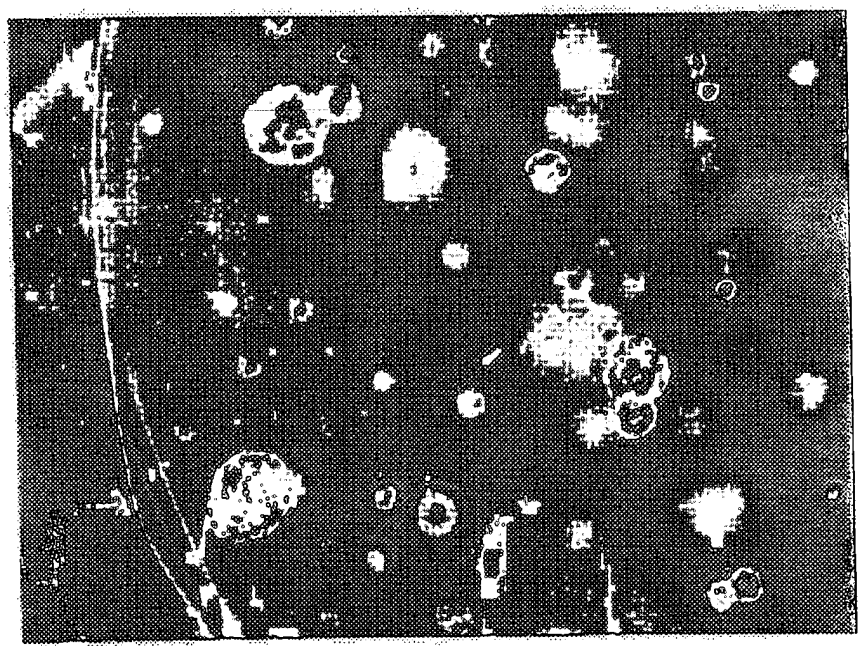

FIG. 6b

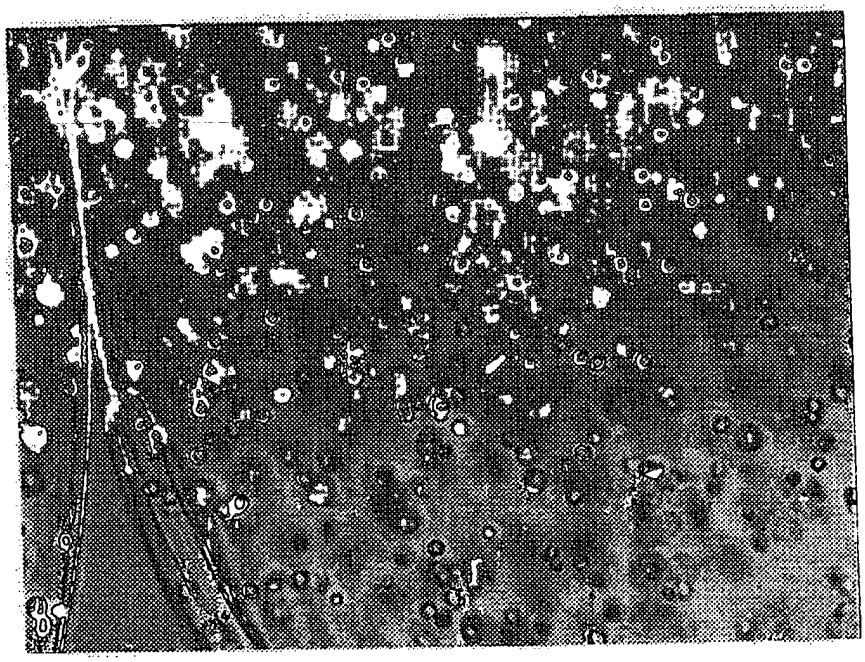

FIG. 6c